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A Study of Enterovirus Isolations in Glasgow From 1977 to 1997

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The number and range of enteroviruses isolated in the Regional Virus Laboratory, Glasgow during 1977–1997 was determined. Over this period, 3,039 enterovirus isolations were reported. The echoviruses represented 67% of isolations with echovirus 4 (due to an outbreak in 1990), echovirus 30 and echovirus 11 being the most frequently isolated types. The pattern of prevalence of non-polio enteroviruses had changed from the previous 20-year period with echovirus types isolated more often (77% vs. 55.4%) and coxsackieviruses isolated less often (23% vs. 44.6%). The polymerase chain reaction (PCR) introduced into the routine diagnostic service in 1996 increased the detection of enteroviruses from cerebrospinal fluid samples compared with traditional cell culture methods. Finally, the 5′ nontranslated region (NTR, bases 63–475) and the VP4/VP2 region (bases 581–1199) of selected echovirus 30 and coxsackie B3 isolates were sequenced. These represented endemic and epidemic types respectively and were shown to be closely related within their type, but different from the published sequences. The current echovirus 30 strains differed from 1966 isolates by 16–20% in both the 5′ NTR and VP4/VP2 regions. The coxsackie B3 isolates, predominant in 1997 after 5 years of absence, were also dissimilar from previously isolated strains, causing a small outbreak. ***J. Med. Virol. 58:304–312, 1999.*** © 1999 Wiley-Liss, Inc.

KEY WORDS: epidemiology; echoviruses; coxsackieviruses; PCR; sequence variation

INTRODUCTION

The Regional Virus Laboratory (RVL) in Glasgow provides a diagnostic virology service and receives approximately 130,000 specimens per year, mainly from the West of Scotland. Of these almost 11,000 samples are for virus isolation studies, yielding herpesviruses, adenoviruses, rhinoviruses, and enteroviruses routinely. The laboratory has a long history of interest in the enterovirus group: a record of 20 years of isolation of selected viruses in Glasgow was reported by Grist et al. [1978]. Enterovirus isolation from clinical samples is frequently unsuccessful due to a number of problems: certain coxsackie A serotypes grow poorly or not at all in cell culture; samples may contain substances toxic to the cells; viral titres may be too low for detection or the virus may not be viable to due delays in transit and specimen handling [Nicholson et al., 1994; Rotbart and Romero, 1995]. In the modern diagnostic laboratory, traditional detection methods are being replaced by molecular techniques such as nucleic acid sequence-based amplification (NASBA) and polymerase chain reaction (PCR). These techniques provide rapid and efficient methods of detecting a number of different agents including the hepatitis viruses [Revets et al., 1996] and the enteroviruses [Rotbart et al., 1994].

This report updates the previous isolation data, with a detailed listing of the enteroviruses reported in the RVL from 1977 to 1997, together providing a record of isolation over the past 41 years. The results of enteroviral PCR testing on samples of cerebrospinal fluid (CSF), which was introduced into the routine diagnostic service in January 1996, are also presented. Sequencing of selected virus isolates was carried out in an attempt to investigate sequence variation over time. Echovirus 30 isolates identified in 1995, 1996, and 1997 were compared with isolates from 1966 and 1975 and a group of coxsackie B3 isolates from 1997 was compared. Coxsackie B3, which was dominant in 1997, had not been isolated in this laboratory since 1991 and 63% of the isolates were from one geographical region, obtained over a short period of time. Two regions of the genome were targets for PCR, part of the 5′ nontranslated region (NTR) and part of the VP4/VP2 region. The amplicons produced were sequenced, compared with each other and with other available sequences, ob-

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tained either from the GenBank database or from "in-house" sequencing of enterovirus isolates.

METHODS

Enterovirus Isolation Data

Samples are cultured for virus on the basis of the clinical information or direct requests from clinicians. All enterovirus isolates are identified in the RVL by traditional cell culture methods [Grist et al., 1979] and typed by standard neutralization assay [Minor and Bell, 1990]. Viruses are isolated from various specimen types including stool, throat swab, nasopharyngeal aspirate, and CSF.

Enterovirus isolation results for the years 1977–1993 were collated from paper records in the laboratory while results for the years 1994–1997 were available via the Telepath computer database. The number of isolates of each virus type from each year was determined and the clinical details associated with the most frequently isolated types were recorded. The age and sex distribution of patients with each enterovirus group was examined and for the years 1994–1997 the monthly distribution of non-polio enterovirus isolations was determined.

Extraction and PCR Amplification

CSF specimens (200 μl) were extracted using TRIzol reagent according to the manufacturers instructions (Gibco BRL), with the addition of ribosomal RNA (75 μg) at the precipitation stage to act as a carrier. The RNA (10 μl) was reverse transcribed to cDNA according to the method described by Galbraith et al. [1995] prior to amplification of the 5′ NTR by a nested PCR using primers P1/P4 and primers P6/P9.

For the first round, a 50-μl reaction mixture was prepared containing 40 pmol each of primers P1+ and P4–, with 5 μl of reaction buffer IV, 3 μl magnesium chloride (25 mM), 1 μl dNTPs (10 mM), and 0.25 μl Thermoprime plus DNA polymerase (5 units/ μl) (all Advanced Biotechnologies). The mixture was overlaid with mineral oil and subjected to the following conditions on a Hybaid Omnigene thermocycler: 1 min at 94°C, followed by 35 cycles of 50 sec at 94°C, 50 sec at 55°C and 1 min, 10 sec at 72°C, ending with a final extension step of 72°C for 10 min.

An aliquot (2 μl) of the first round product was the amplified in a 50-μl reaction (1.5 mM magnesium chloride) containing primers P6+ and P9–. Reaction conditions were as for the first round, except that 25 cycles were performed. The nested primers amplified all the enteroviruses (59 types) available for testing in the laboratory with the exception of echovirus type 22. Coxsackievirus types A6 and A24 and echovirus types 23, 29, and 32 were not available for testing.

In addition, the cDNA was tested with primers ABL1/ABL2. These amplify constitutive ableson tyrosine kinase mRNA and give rise to a positive band in all human RNA. This PCR therefore controls for the quality of RNA and successful amplification [Gow et al., 1991].

Virus positive cell-culture fluid was extracted in the same manner but was subjected to one round of PCR only either with primers P1+ and P4– (for the 5′ NTR) or primers A+ and B– for amplification of the VP4/VP2 region. The latter PCR was carried out as for the first round except that the annealing temperature was 60°C instead of 55°C. The A+/B– primer combination detects the majority of enterovirus types with the exception of coxsackie A types 2, 4, 8, 12, 14, and 16, and echovirus type 22. As above, some types were unavailable for testing.

The PCR products were visualised by ethidium bromide staining in 1.5% agarose gels. All experiments were conducted with positive and negative controls.

Primers

P1+ 5′ CGGTACCTTTGTGCGCCTGT (63–82)
P4- 5′ TTAGGATTAGCCGCATTCAG (475–457)
P6+ 5′ GCACTTCTGTTACCCC (168–183)
P9- 5′ TCAATAGACTCTTCGCAC (433–416)
A+ 5′ TGGCTGCTTATGGTGACAAT (581–600)
B- 5′ TCTGGGAACTTCCACCACCA (1199–1180)
ABL 1 5′ CAGCGGCCAGTAGCATCTGACTT (N/A)
ABL 2 5′ TGTGATTATAGCCTAAGACCCGGAG (N/A)

Nucleotide positions (in parentheses) are relative to coxsackievirus B3 [Klump et al., 1990].

Sequencing

Unincorporated nucleotides and reagents were removed from PCR products by centrifugation through a Microspin S-400 column (Pharmacia). The products were then sequenced in the laboratory (with either P1+/P4– or A+/B– primers) using dye terminator cycle sequencing (ABI prism system, Applied Biosystems). Electrophoresis of products was carried out on an ABI 373 Automated Sequencer by staff of the Molecular Biology Support Unit, University of Glasgow. The derived sequences were analysed using the Genetics Computer Group (GCG) software package (University of Wisconsin) [Devereux et al., 1984]. Similarities between individual sequences were determined using the program GAP. Dendrograms were constructed using the program PILEUP, followed by DISTANCES and GROWTREE.

RESULTS

Enterovirus Isolates

Table I shows the enterovirus isolates identified and typed in the RVL from 1977 to 1997. Over the time period studied, 3,039 enteroviruses were typed. The echoviruses as a group, represented 67% of the isolations with, in decreasing order, echovirus 4, echovirus 30, echovirus 11, echovirus 7, and echovirus 9 as the most frequently isolated virus types. Coxsackie B viruses accounted for 14.2% of isolates and 5.6% belonged to the coxsackie A group (coxsackie A9 accounting for 77% of these). No wild-type indigenous poliovirus was isolated during this 21-year period, all 391 isolates (which comprised 13% of all isolations) arising

TABLE I. Enterovirus Isolates Identified by the Regional Virus Laboratory During 1977–1997

Virus type	1977	1978	1979	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997
Poliovirus 1	1	5	2	3	8	3	2	3	2	9	5	8	7	8	15	13	10	12	12	12	11
Poliovirus 2	3	4	3	13	4	7	3	3	3	13	5	5	3	5	10	8	13	17	2	7	4
Poliovirus 3	1	2	0	8	5	2	2	6	2	5	3	11	3	1	7	10	11	8	6	4	8
Coxsackie A9	0	0	2	6	3	3	5	3	2	2	13	6	4	13	2	10	**19**	5	**22**	6	4
Coxsackie A10	0	0	1	0	0	2	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0
Coxsackie A16	2	0	0	8	1	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0
Coxsackie A21	0	0	0	0	0	0	0	1	0	0	0	3	3	0	0	0	0	1	0	0	0
Cox A untyped	0	0	0	3	0	0	7	0	0	0	1	0	0	1	0	0	0	0	0	0	0
Coxsackie B1	2	0	0	0	0	3	**14**	1	0	0	0	0	3	3	5	1	6	15	0	0	0
Coxsackie B2	0	5	3	0	18	6	0	3	1	1	**16**	2	1	4	0	3	2	2	14	1	0
Cosackie B3	0	1	0	0	1	1	2	14	22	4	9	1	1	2	6	0	0	0	0	0	**19**
Coxsackie B4	0	0	**10**	1	4	6	2	4	14	2	0	8	0	1	3	8	6	15	2	13	2
Coxsackie B5	5	0	0	4	3	0	0	**22**	6	6	1	0	4	6	9	12	1	2	1	**30**	0
Coxsackie B6	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
ECHOvirus 3	4	8	0	0	0	6	1	1	2	1	5	0	2	0	0	4	3	0	0	0	0
ECHOvirus 4	1	0	0	0	0	0	0	1	1	2	2	0	1	**452**	18	0	0	1	0	0	0
ECHOvirus 5	1	0	0	0	0	5	8	4	**24**	5	0	5	1	10	1	0	0	4	2	0	0
ECHOvirus 6	**8**	1	0	1	6	3	**13**	2	2	5	3	**35**	4	0	0	0	2	**30**	5	0	6
ECHOvirus 7	0	4	**13**	15	6	1	0	**27**	18	0	0	0	**35**	0	0	2	0	12	5	2	9
ECHOvirus 9	7	0	1	4	8	15	3	6	4	**27**	4	7	6	1	6	4	10	7	16	1	1
ECHOvirus 11	2	**28**	4	1	1	**95**	2	16	**32**	19	2	13	**19**	1	1	**16**	1	2	14	5	1
ECHOvirus 17	0	2	4	1	18	**71**	2	0	0	0	0	0	1	4	7	3	0	0	1	0	0
ECHOvirus 18	0	0	0	0	4	1	0	0	0	3	3	0	0	0	25	0	0	0	0	1	1
ECHOvirus 22	0	0	0	3	1	0	3	1	1	1	**21**	7	0	3	1	1	3	5	1	0	0
ECHOvirus 30	0	0	2	**147**	**38**	5	1	4	9	21	14	9	4	3	**63**	15	0	0	8	**27**	6
ECHOvirus other	7	11	22	6	15	7	7	5	4	9	2	5	10	5	7	0	2	15	4	6	0
Enterovirus untyped	5	0	0	18	0	0	1	0	0	3	0	0	0	2	0	0	0	3	3	1	7
TOTAL	49	71	68	242	144	242	78	128	149	139	109	128	113	525	186	110	89	156	118	116	79

Enteroviruses representing greater than 15% of the total for that year are highlighted. Those echoviruses with fewer than 20 isolates over the study period are grouped as “echovirus other”.

TABLE II. Clinical Symptoms (%) Associated With the Nine Most Frequently Isolated Enterovirus Types (Excluding Coxsackie A9)

Virus type/symptoms	Cx A9	Cx B4	Cx B5	Echo 6	Echo 7	Echo 9	Echo 11	Echo 17	Echo 30
Neurological	61	30	59	62	60	69	63	72	82
Pyrexia	18	25	16	18.5	17.7	20	13	5.3	6.8
Respiratory	13.5	30	19	17	29	15.6	22	18	7.7
Gastrointestinal	10	20.5	10	21	10.6	15.6	10	10.6	12.4
Musculoskeletal	0.8	4.8	5.7	2	0	2.7	1	2.1	.06
Rash	13.5	2.4	2.8	8.2	5.3	19.3	4.4	4.2	1.8
Sudden infant death (SID)	1.7	4.8	0.9	0	2.6	0	1.7	0	0.3
Failure to thrive (FTT)	0	4.8	0.9	1.0	0	0	1.7	2.1	0.3
Eye infection	0	0	0	0	0	0	0.4	0	0
Cardiac	0	1.2	0.9	1.0	0	0	0.4	1.0	0

from vaccine-derived or imported cases. Each year 9 (1997) to 23 (1989) different non-polio enteroviruses were isolated and one or two types were seen to be predominant. The most striking feature was the echovirus type 4 outbreak in 1990, which accounted for 86% of the enterovirus isolates that year [Gallacher et al., 1993]. This finding contrasts with the previous 13 years of this study period, in which only 8 isolates of this virus were identified.

The monthly distribution of the non-polio enterovirus isolates for the years 1994–1997 was determined. Coxsackie B virus activity peaked in July and August, with no isolates in January or February. Echoviruses were present throughout the year peaking in the months of July–October. Coxsackie A virus types were also reported throughout the year albeit in small numbers with approximately half of all isolates detected in July and August.

The majority of the coxsackieviruses were isolated from children under 14 years of age (77% and 83% for types A and B, respectively) with a male-to-female ratio of 1.5:1. Echoviruses were not over represented in any age group, although only 8% were isolated in individuals over the age of 30. The male-to-female ratio was approximately 1.4:1.

The clinical details associated with the nine most frequently isolated serotypes (excluding echovirus type 4) were grouped into categories. For example, meningism, headache, and photophobia were classed as neurological symptoms, diarrhoea and vomiting as gastrointestinal symptoms (Table II). The symptoms were not mutually exclusive and thus the percentages for each virus type add up to more than 100. For all the serotypes, with the exception of coxsackie B4, neurological symptoms were dominant, occurring in 60–82% of cases. In the case of coxsackie B4, neurological symptoms were reported in only 30% of cases, with respiratory, gastrointestinal, and pyrexial symptoms reported as often. Musculoskeletal symptoms, including myalgia and chest pain, were associated more often with coxsackie B4 and B5 types and only one case of conjunctivitis was recorded with echovirus 11 isolated. Isolations of a number of different enteroviruses from a small number of cases of sudden infant death were also noted.

In 1990, isolates of echovirus type 4 were reported from February to December with 32% isolated in September. Symptoms of meningitis were present in 98% of cases and 80% of the viruses were isolated from individuals aged between 5 and 29 years. The male-to-female ratio was approximately 1:1.

PCR Testing Versus Culture for CSF Specimens

From January 1996 to May 1998, 887 CSF specimens from the routine service were tested for the presence of enteroviral sequences by nested PCR. Of these, 237 (17 of which were PCR positive) had insufficient volume for subsequent viral culture and 4 (0.5%) were ABL-PCR negative. Of the remaining 646, 56 (8.6%) were enteroviral PCR positive and 590 were negative (Table III). Concordant results were observed in 596 samples, whereas 48 were PCR positive/culture negative and two were PCR negative/culture positive. These two specimens were slow to grow in culture and were eventually identified as coxsackie A9 types.

Sequence Comparison

Eight echovirus 30 isolates from between 1966 and 1997 were amplified successfully by both sets of primers. Details of patients and symptoms are given in Table IV. Readable sequence from both the 5′ NTR and the VP4/VP2 regions was obtained from six, and the two others provided sequence for one or the other region only. Nine coxsackie B3 isolates, detected over a 5-month period in 1997, were also amplified successfully and sequenced using both sets of primers. The sample denoted 1996 B3QC was received in the laboratory as a quality control specimen from the public health laboratory, Colindale, and was used as a comparison sequence.

TABLE III. Comparison of Enterovirus PCR and Culture Results

Test result	Enterovirus culture positive	Enterovirus culture negative	Total
Enterovirus PCR positive	8	48	56
Enterovirus PCR negative	3	588	591
Total	11	636	647

PCR, polymerase chain reaction.

TABLE IV. Selected Coxsackie B3 and Echovirus Isolates Identified by the RVL

Isolate	Isolate number	Sex	Age	Location	Date sample received	Clinical details
Coxsackie B3	3629	F	18 months	DRI	07.05.97	Pyrexia
	3748	M	7 years	DRI	12.05.97	Sore throat, tonsilitis
	4413	M	2 years	DRI	03.06.97	Pyrexia
	4541	M	6 years	DRI	07.06.97	Pyrexia, vomiting
	4625	M	4 years	DRI	11.06.97	Pyrexia
	5027	M	3 years	DRI	24.06.97	Febrile convulsions
	6220	M	1 month	DRI	01.08.97	Pyrexia unknown origin
	6687	M	4 years	RHSC	19.08.97	No details
	8985	M	2 years	SRI	22.10.97	No details
	1996B3QC	U	12 weeks	CPHL	26.06.96	Pyrexia
Echovirus 30	6392	F	12 years	Ayrshire	07.66	Sore throat, meningitis
	6398	M	1 year	Ayrshire	07.66	Meningitis
	6548	F	11 years	AA	11.75	Meningitis
	8117	M	7 months	MDGH	26.10.95	Meningitis
	8293	M	5 years	MDGH	01.11.95	Headache, meningitis
	3786	M	8 years	DRI	21.05.96	Headache
	4313	M	15 years	DRI	07.06.96	Meningitis
	4844	F	9 months	RHSC	17.06.97	Fit

RVL, Regional Virus Laboratory; DRI, Dumfries Royal Infirmary; RHSC, Royal Hospital for Sick Children, Glasgow; SRI, Stirling Royal Infirmary; MDGH, Monklands District General Hospital; AA, Ayrshire and Arran; 1996B3QC, sample received from Central Public Health Laboratory (CPHL) Colindale, for quality control testing.

1. The 5′ nontranslated region. A dendrogram depicting the relationships of the echovirus 30 and the coxsackie B3 isolates within the enterovirus group is shown in Figure 1. Two clusters are observed: one containing the polioviruses, coxsackie A virus types A15, A21, and A24, and enterovirus 70, the other containing the remainder of the sequences including those of the echoviruses. The echovirus 30 isolates fall into two groups in the dendrogram, the isolates from 1966 and 1975 (echo 30 6392, 6398, and 6548) clustering apart from the 1995–1997 isolates (echo 30 3786, 8117, 4844, and 4313). The earliest isolate (6392) was 97.3% similar to the second 1966 (6398) isolate and 92.4% similar to the isolate from 1975 (6548). Similarities of 6392 with the 1995–1997 strains ranged from 84.2% to 87.7%. Sequence variation between the two clusters was not confined to any particular region but was spread throughout the sequence. Within the recent isolate cluster the similarities were approximately 97%.

The seven clinical isolates differed from the published echovirus 30 sequence (echo30fin) isolated in 1985, by up to 17%.

The nine coxsackie B3 isolates from 1997 clustered closely together on the dendrogram, with similarities between 97.2% and 100%. Isolates 3629, 3748, 4541, 4625, and 5027 (all from Dumfries Royal Infirmary [DRI]) were identical in this region. Isolate 4413 was greater than 98.0% similar to the other DRI isolates as was isolate 6220 taken 2 months later. The isolates from Royal Hospital for Sick Children (RHSC) (6687) and Stirling Royal Infirmary (SRI) (8985) were also closely related to those from DRI in this region with similarities of more than 97%. Compared with the QC isolate (which was 100% identical to the published coxsackie B3 sequences cox B3A and B), the 1997 B3 isolates differed by up to 17%.

2. The VP4/VP2 region. A dendrogram depicting the relationships of the echovirus 30 and coxsackie B3 isolates in the VP4/VP2 region is shown in Figure 2. In this region, the seven echovirus type 30 isolates clustered together with similarities between 79% and 87%. The most closely related isolates (8117 and 8293) were from samples obtained within 6 days of each other from the same hospital. Isolates 3786 and 4313, obtained from a different hospital 17 days apart, were 95% similar to each other.

The 1997 B3 isolates clustered together with similarities between 91.8% and 99.3%. Compared with the QC isolate (92 to 95% similar to the published coxsackie B3s A, B, and C), the 1997 B3 isolates differed by up to 27%.

DISCUSSION

This report updates previous data from the Regional Virus Laboratory in Glasgow on the isolation and characterization of enteroviruses [Grist et al., 1978]. During this period, the population remained largely unaltered and the catchment area continued to be the West of Scotland. Virus isolation procedures have remained virtually unchanged except for the cessation of the use of suckling mice; thus comparison of virus isolation results over the 41 years is valid.

Enterovirus Isolates

In temperate climates, the majority of non-polio enterovirus (NPEV) isolations occur during the summer and autumn months in comparison to tropical climates where enteroviruses are isolated throughout the year. Vaccine-strain poliovirus is also isolated throughout the year in temperate climates where there is an immunisation programme for the administration of oral poliovirus vaccine. In the United States, 82% of enteroviruses were isolated during the months of June–October [Moore, 1982]. In the present study, for the

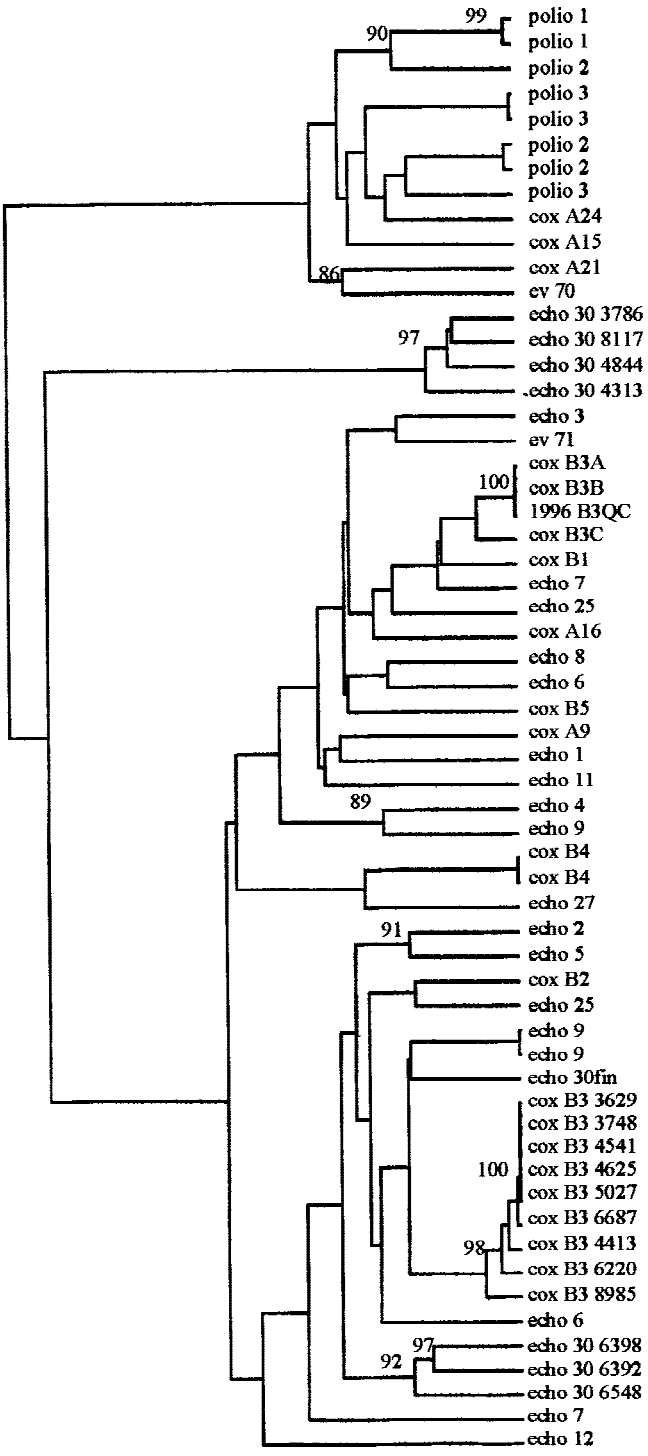

Fig. 1. Dendrogram showing the relationship of the echovirus 30 and coxsackie B3 isolates within the enterovirus group based on the 5′ nontranslated region (NTR). The numbers indicate the percentage similarity at that point.

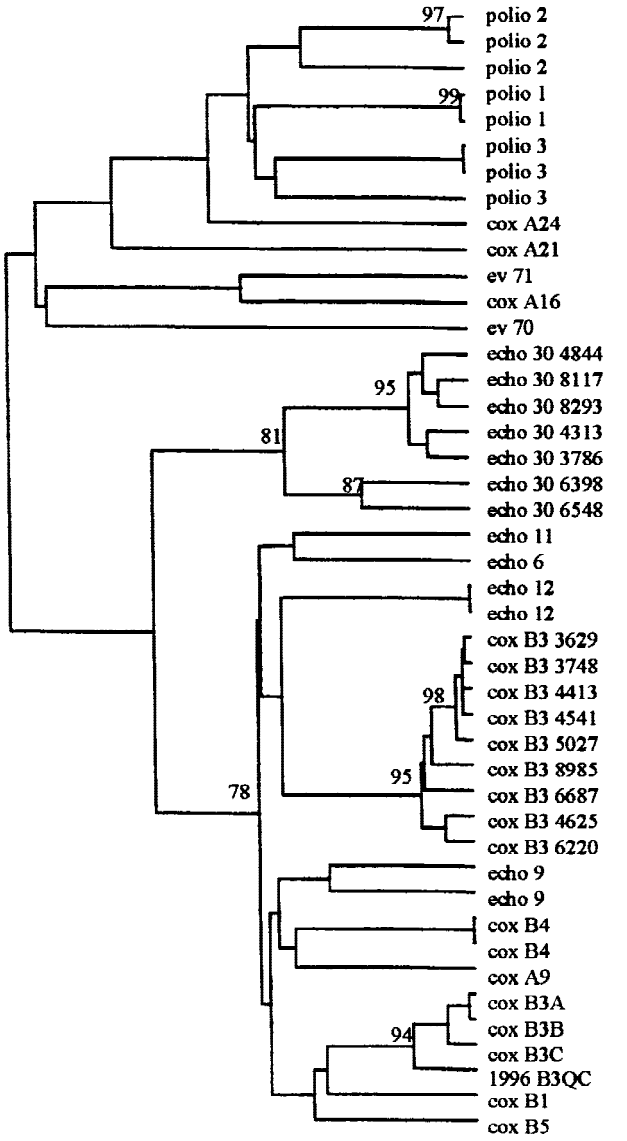

Fig. 2. Dendrogram showing the relationship of the echovirus 30 and coxsackie B3 isolates within the enterovirus group based on the VP4/VP2region. The numbers indicate the percentage similarity at that point.

years 1994–1997, NPEV isolation peaked in the months of July and August, with 73% of isolates occurring in the months of June–October.

Data from the World Health Organisation on enterovirus isolation worldwide has been reported previously [Assaad and Cockburn, 1972; Grist et al., 1978; Grist and Reid, 1988]. From 1967 to 1983, the pattern of prevalence of NPEV changed with echoviruses isolated more often and coxsackie A and B types isolated less often. The data in this report, describing the years 1977–1997, support this trend, with echoviruses representing 77% of isolates and coxsackie A and B types accounting for 23%. These results contrast with the data for the period 1967–1974, with 55.4% echoviruses and 44.6% coxsackie A and B types reported [Grist et al., 1978]. Within the coxsackie A and the echovirus group, however, the actual virus types isolated have not altered to any great extent, with coxsackie types 9 and 16 and echovirus types 9, 11, and 30 still the most frequently isolated enteroviruses.

A previous report of enterovirus isolations in

Glasgow showed the cyclic recurrence of certain virus types over 20 years of observation [Grist et al., 1978]. In any one year, 25 or more different echovirus types were isolated, but some types showed a tendency to recur. Echovirus 9, for example, recurred at 4-year intervals. This pattern was not repeated in the recent 21-year period. Echovirus 9 occurred at a steady rate with isolates every year (apart from 1978) with no particular increase in occurrence. Echovirus 9 was the predominant virus in 1986, but with only 20% of the total isolates.

In the 1957–1976 period, echovirus types 4 and 30 were absent for 7–9 years between outbreaks. In this report, echovirus 30 was isolated almost every year at low levels with epidemics in 1980–1981 and 1991. However, with echovirus type 4, there were only 8 isolates in 13 years before a large outbreak in 1990–1991. After this, the number dropped again with only one isolate in the next 6 years.

Echovirus 17 was reported more often in this 21-year period than in 1957–1976 with 114 isolates (62% of which came from 1982) compared with 26. Other echovirus types exhibited cycling in terms of predominance: echovirus 6 was the predominant virus type every 5–6 years, whereas echovirus 11 was predominant every 3–4 years.

The coxsackie B viruses were isolated with varying frequency over the recent 21-year period. In some years, five serotypes were evident (1984 and 1990), whereas in others only two serotypes were reported (1980). With the exception of coxsackie A9 there were few coxsackie A isolates, probably due to the inability of many types to grow in traditional cell culture systems combined with the cessation of isolation in newborn mice in the mid-1980s. Most of the other enteroviruses did not show any particular pattern of occurrence throughout the study period.

Isolation data has also been described from Belgium for the period 1980–1994 [Druyts-Voets, 1997]. Three of five of the most frequently isolated non-polio enteroviruses were also isolated most often in Glasgow (echovirus types 7, 11, and 30). However, the Belgian data showed that coxsackie B virus isolates accounted for a greater proportion of NPEV isolates than in Glasgow (22.4% versus 16.4%), with only a few coxsackie B6 virus isolates reported. Data from England and Wales for the period 1975–1994 from more than 40,000 confirmed isolates also showed that coxsackieviruses accounted for a greater proportion of NPEV (29%) [Atkinson et al., 1998]. In all cases, coxsackie A viruses accounted for the smallest proportion of isolates.

In Belgium, echovirus 30 was the most frequently isolated virus type with peaks in 1980, 1988, and 1992. There appear to be comparable outbreaks in Glasgow in 1980 and 1991–1992, but not in 1988. Echovirus 30 was also the most important agent associated with aseptic meningitis in Japan, causing an outbreak in 1989–1991 with more than 5,000 isolations [Anon., 1998].

Echovirus 33, which caused outbreaks in France, the Netherlands, and Belgium in 1982, was not isolated in Glasgow that year, with only 17 isolates in total in the 21-year period. The echovirus 4 outbreak observed in Glasgow was not reported elsewhere.

The Belgian report also noted differences in the ages of patients with different virus types. Coxsackie A and B infections were most frequent in the 1–4-year-old age group and echoviruses in the <1-year-old age group. This was not the case with the Glasgow data, in which the majority of coxsackie viruses were isolated in children younger than 14 years and echoviruses isolated in all ages. A male-to-female preponderance of 1.5:1 to 2.5:1 noted by others was also recorded in this study. Certain echovirus types are important in early infancy, including echovirus 4 in febrile illnesses, echovirus 9 in fatal and mild infection, and echovirus 11 in meningitis, respiratory and gastrointestinal illnesses [Modlin, 1986; Druyts-Voets, 1997]. In the current report, different serotypes were not particularly associated with specific symptoms, with the exception of echovirus type 4, which was associated with symptoms of meningitis in 98% of cases. In Belgium, 63% of echovirus 4 isolates were associated with symptoms of meningitis. The first outbreak of echovirus 4 in Britain (also reported from this laboratory) was also mainly associated with meningitis and had an older age distribution [Bell, 1964].

Coxsackie B viruses are important agents of acute heart disease [Muir, 1993], but in this study symptoms of cardiac involvement were evident in only a few cases. This is because testing of samples from such patients in this laboratory is performed by serological methods.

PCR Testing

Previous data has indicated that 25–35% of specimens from patients with characteristic enterovirus infection will be negative by cell culture [Chonmaitree et al., 1982]. Thus, PCR has emerged as a method of detecting viral sequences that is not subject to many of the limitations of cell culture, which includes the virus being neutralised by antibody or the sample being toxic to the cells.

A previous report from this laboratory described the detection of enterovirus in clinical samples from patients with aseptic meningitis during 1994 and 1995 [Riding et al., 1996]. An increase in positivity from 6% (by cell culture) to 27% using nested PCR was shown. This has been demonstrated by a number of groups [Rotbart, 1990; Johnston et al., 1993; Leparc et al., 1994; Yerly et al., 1996]. This prompted the introduction of PCR screening of CSF samples into the routine diagnostic service in January 1996, followed by cell culture if sufficient sample remained. This report presents the results of real-time testing on a large number of samples.

In this study, testing by PCR was shown to be more sensitive than cell culture alone. However, the primers used do not detect echovirus type 22, which has been shown to be an atypical enterovirus, as has echovirus 23 (not available for testing) and thus without culture these types will be missed. The primers do, however,

pick up the coxsackie A virus types that will not grow in cell culture, which is an improvement. Over the time period studied, only two samples were shown to be false negatives by PCR. It may be that these were on the threshold of sensitivity for detection by PCR.

A small proportion (0.5%) of samples were ABL-PCR negative, prompting the request for a second specimen. This suggests that the RNA may have degraded during processing or perhaps there were inhibitors of the PCR process in the sample.

Echovirus 30 and Coxsackie B3 Isolates

Phylogenetic analysis of the 5′ NTR sequences and capsid protein sequences has been described elsewhere (Hyypia et al., 1997). Analysis of the 5′ NTR showed the presence of two clusters of sequences, as was demonstrated here. Analysis of the VP2 capsid sequences indicated the presence of four clusters: A (coxsackie A types and enterovirus 71), B (coxsackie B types, echoviruses, coxsackie A9, and enterovirus 69), C (polioviruses and coxsackie A types), and D (enteroviruses 68 and 70). Analysis of the VP4/VP2 region in the present study did not include any of the group A sequences and only one of group D. Thus enterovirus 71, coxsackie A16, and enterovirus 70 aligned with the group C sequences. All those identified as group B sequences by Hyypia and colleagues clustered together in the present study.

Echovirus 30 represented a virus type isolated almost every year since 1979. Analysis of the conserved 5′ NTR sequence showed that isolates closely related in terms of sequence similarity were closely related for the time of isolation with sequences from 1966 and 1975 clustering apart from the 1995–1997 isolate sequences. More specifically, comparing the first isolate in 1966 with the other six, the closest in similarity was the second 1966 isolate and the least similar was that from 1997, with a difference of 16%. However, sequence from the early isolates was more similar to that from other serotypes (i.e., 92% similar to coxsackievirus B3) than to their own serotype, thus accounting for the two clusters. In the capsid region, the echovirus 30 sequences clustered together: those isolates obtained within a short time span (4313 and 3786, 8117 and 8293) were more similar to each other than to those isolated years apart. Although the similarities within the echovirus 30 group vary from 80% to 95% and are similar to those observed for the 5′ NTR sequences, they are different from the other serotypes in the VP4/VP2 region and thus cluster together.

The 1997 coxsackie B3 isolates also showed high degrees of similarity in both regions, with five of them (all from DRI) 100% identical in the 5′ NTR. When identical sequences are observed the question of contamination with PCR products arises. All reasonable precautions are taken in the laboratory to minimize the risk of contamination. These precautions include the physical separation of pre- and post-PCR laboratories, use of dedicated pipettes at each stage, and inclusion of positive and negative controls. Although five samples were identical in the 5′ NTR sequence, there was no evidence of contamination. Positive and negative controls gave the expected results and sequence from the VP4/VP2 region of the five samples was not identical showing that they were independent. Compared with a QC isolate, tested in the laboratory in the previous year, the 1997 isolates differed by approximately 15% in the 5′ NTR and up to 27% in the VP4/VP2 region. The sequence information therefore showed that the coxsackie B3 isolates were significantly different from previously sequenced isolates and this may have been why they caused a small outbreak after an absence of 5 years.

High sequence identity among echovirus type 30 isolates has been reported previously [Drebot et al., 1994]. Sixty isolates from seven different Canadian provinces in 1991 were found to be more than 99% similar for the 5′ NTR, with the majority of sequences identical. Additionally, a series of coxsackie B1 isolates from 1992 proved identical in this region.

Echoviruses isolated in different years (1988–1993) have been shown to have homology exceeding 98% [Diedrich et al., 1995]. In both reports, the differences between current clinical isolates and prototype strains were highlighted. This was also noted in the current study and can be a problem when reference strains are used for neutralization assays. It may be necessary to use different strains in the future if the trend in variation continues.

This report is important in extending the previous work on enteroviruses producing overall, a continuous 41-year history of enterovirus isolations from the Regional Virus Laboratory in Glasgow. The cycling nature of some enteroviruses has been shown but the reasons for this are unknown. Outbreaks of certain serotypes can occur in different countries at the same time and they can also occur in one country whilst being absent in another. It would be interesting to compare the sequence of isolates from outbreaks in different countries to see if there was a pattern. The type of enteroviruses encountered over the years has not varied a great deal, but the proportion of coxsackievirus isolates has decreased while echovirus isolations have increased. Additionally, modern molecular techniques have been shown to be useful in the routine diagnostic laboratory, with higher detection rates than traditional methods, suggesting that the number of isolations underestimate the prevalence of the enteroviruses. They also show that there may be considerable sequence divergence over time within a particular serotype.

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